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Amino acids attached to transfer RNA during the vegetative growth of *Cenococcum geophilum*

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INTRODUCTION

Alterations in the levels of specific transfer RNA (tRNA) species have been observed in association with changes in the rate of specific protein synthesis and appear to play an important role in cell differentiation (5). Before studying the alterations in tRNA concentrations in ectomycorrhizal tissues during early infection, we have needed to develop a direct method for determination of amino acids attached to tRNA in vivo. The method developed was tested by following the levels of amino acyl-tRNA during the growth of the ectomycorrhizal ascomycete *Cenococcum geophilum*.

The determination of amino acids attached to tRNA in vivo involves two major difficulties. One is the isolation and purification of tRNA from the source material under conditions that avoid hydrolysis of the amino acyl-tRNA ester bond. Another is the separation and quantification of small amounts of amino acids obtained from tRNA by deacylation.

MATERIAL AND METHODS

Growth of the fungus. *Cenococcum geophilum* Ferd. and Winge, strain Kiffer 1973 was grown as described previously (4b).

tRNA preparation. The method was a modification of previously published methods (2, 3, 7). Mycelia were collected by filtration and immediatly frozen in liquid nitrogen. Conditions were selected to minimize any possible deacylation of the tRNA during the stages of its isolation and purification. It has been shown that the most labile ester bond is stable at pH 5.1 and extraction was therefore carried out at pH 5.0.

Stage 1. Mycelia (20g) were homogenized for 1 min in a Waring blender with 100 ml of a solution containing 1mM sodium acetate buffer at pH 5.0, 5 mM NaCl, 0.1 mM $MgCl_2$, 0.5 % (w/v) sodium dodecylsulfate, 2% (w/v) Triton X 100 and 1mM cycloheximide (buffer 1) and 100 ml of buffer-saturated phenol containing 14% (v/v) m-cresol and 0.1 % (w/v) 8-hydroxyquinoline. The mixture was shaken at room temperature for 10 min. The homogenate was centrifuged at 12,000 g for 15 min and the upper aqueous layer removed for stage 2. The phenol phase was re-extracted with one volume of 1mM sodium acetate, 5mM NaCl, 0.1 mM $MgCl_2$ at pH 5.0. The resulting aqueous phase

was combined with the aqueous phase from the first extraction and one additional phenol extraction was performed for 10 min at room temperature.

Stage 2. The nucleic acids were precipitated from the pooled aqueous phases by addition of 0.1 volume of 20% (w/v) potassium acetate, pH 4.9 (buffer 2) and 2 volumes of cold ethanol. After mixing, the white flocculent material was allowed to precipitate overnight at -20°C. After centrifugation at 27,000g for 15 min, the supernatant was discarded and the precipitate dried with a stream of N_2. Traces of ethanol remaining caused difficulties in solubilizing the precipitate at the next stage.

Stage 3. tRNA was purified by gel permeation. The nucleic acid precipitate was resuspended in 5mM sodium acetate buffer, pH 5.0, containing 500 mM NaCl and 10 mM $MgCl_2$ (buffer 3) and loaded on a column (60 cm x 1.8 cm) of Fractogel TSK W55 (superfine) (Merck) at 4°C. The eluting buffer (buffer 3) was passed through the column at 50ml/h and monitored with a Shimadzu spectrometer at 260 nm. Fractions containing tRNA (5 ml) were collected pooled and tRNA was precipitated overnight by addition of 0.1 volume of buffer 2 and of 2 volumes of cold ethanol. When needed, tRNA was redissolved in buffer 3 and the absorbance units measured at 260 nm. One A_{260} unit of tRNA is defined as the quantity which, when dissolved in 1ml, gives a solution having an absorbance of 1 at a path length of 1 cm, at a wavelength of 260 nm. This corresponds to 62.5 µg of purified yeast tRNA (Boerhinger).

Deacylation of aminoacyl-tRNA. The aminoacyl-tRNA species are alkali-labile (1). RNA samples were resuspended in buffer 3 and amino acids were released from the tRNA by adjusting the pH to 9.5 with 500 mM NH_4OH. The mixture was incubated at 37°C for 90 min. Precipitation of deacylated tRNA was achieved by addition of cold ethanol (2 volumes) and centrifugation at 27,000g for 15 min. The deacylated tRNA was retained for polyacrylamide gel electropheresis analysis. The supernatant was concentrated and analysed for amino acids by high performance liquid chromatography.

Preparation of samples for high performance liquid chromatography. The dried samples were dissolved in 0.01 HCl and this solution was passed through a small column (5cm x 1cm) packed with Dowex 50 W-X8 (H^+) cation-exchange resin. The resin was washed with 25 ml of distilled water and the amino acids were eluted with 20 ml of 6N NH_4OH. The eluate was taken to dryness.

Amino acid analysis. Amino acids were analyzed by high performance liquid chromatography (HPLC) as described previously (4) using Altex-Beckman equipment. Briefly, amino acids were derivatized with ortho-phthaldialdehyde for 1 min and separated by reverse-phase chromatography on C_{18} Ultrasphere ODS column (Beckman Instruments). Derivatives were eluted by using a methanol gradient and detected at 340 nm.
From 20g of wet mycelia, the amino acids from the isolated tRNA were dissolved in 1ml of distilled water and 200 µl samples were used for the derivatization and injected on to the HPLC column.

RESULTS AND DISCUSSION

tRNA isolation. Cycloheximide treatment, rapid freezing of the mycelia in liquid N_2, and handling of the tRNA throughout the purification scheme in solutions buffered to pH 5.0 were designed to inhibit elongation reactions on ribosomes during sample collection and also non-enzymatic

deacylation of tRNA during purification (1,2,3). The phenol-extracted tRNA eluted as a symetric peak on Fractogel TSK W 55 gel filtration (not shown). RNA samples were chromatographed in two portions to ensure that RNA species of higher molecular mass (rRNA and mRNA) were not mixed with the tRNA peak during the gel filtration. The average yield of tRNA obtained from 100g of wet mycelia (14 to 17 day-old) was 25.0 $\pm$ 2.7 mg (n=4).

The tRNA content was highest during the early exponential phase of growth (Fig. 1) and then, regularly decreased during the late exponential phase and stationary phase of growth.

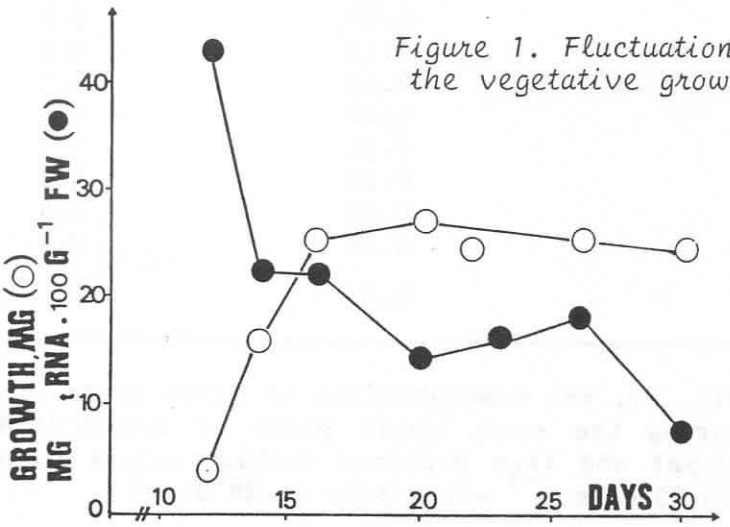

Figure 1. Fluctuations of the tRNA content during the vegetative growth of Cenococcum graniforme

The tRNA content compares well with the value of 12 mg g^{-1} fresh weight reported for rabbit liver (1,2) *Spirodela polyrhiza* (3), and the Deuteromycete *Cylindrosporum ianthothele* (6).

Amino acids attached to tRNA. Table I presents the results for the mol percentage distribution of 14 amino acids attached to *C. geophilum* tRNA in vivo. The values for glutamate, alanine and glycine were particularly high. These amino acids represented 57 % of the total amino acyl-tRNA. The relative scarcity of serine, methionine, and asparagine and the absence of glutamine were especially noteworthy. Proline, hydroxyproline and cystine were not detected by using ortho-phthaldialdehyde derivatization of amino acids.

The average yield of 25.0 mg of tRNA (molecular mass 25,000) obtained from 100g of mycelia was equivalent to 1.0 µmol. The mol percentage values of amino acids attached to tRNA in vivo (table I, column 3) were converted into the absolute values, nmol of tRNA amino acids per mol of tRNA (table I, column 1) and per g of wet weight (table I, column 2). A total of 656 nmol of amino acids were attached to 1 µmole of tRNA. This represented about 66% charging for 14 amino acids in vivo. This value is in agreement with those reported for other organisms (1,2). The nature of the inactive tRNA is not known but it may consist of incomplete tRNA molecules or RNA molecules other than tRNA (1). The total amino acyl-tRNA content was 5.8 nmol g^{-1} wet weight and the amount of amino acids in the free pool was 1×10^3 times greater (not shown). No significant correlation was observed with quantities of free amino acids in *C. geophilum* and amino acids attached to tRNA.

Table 1. Amino acids attached to Cenococcum geophilum (16 day-old) tRNA in vivo

	tRNA amino acids (nmol μmol^{-1} of tRNA)	(nmol g^{-1} of mycelia)	mol %
Asp	39.5	0.31	5.4
Glu	198.8	1.76	30.5
Asn	19.3	0.17	2.9
Ser	10.4	0.09	1.5
Gly	81.2	0.71	12.3
Arg	32.8	0.29	5.0
Ala	91.1	0.80	13.9
Tyr	22.3	0.19	3.3
Met	14.0	0.12	2.1
Val	30.0	0.26	4.5
Phe	35.2	0.31	5.4
Ile	31.2	0.27	4.7
Leu	35.9	0.31	5.4
Lys	20.7	0.18	3.1
TOTAL	658.8	5.77	100

As shown for tRNA content (Fig. 1), the concentration of amino acids attached to tRNA was highest during the early rapid phase of growth (5.8 nmol g^{-1} wet weight at 16 days) and then declined to lower values during the stationary phase of growth (3.0 nm g^{-1} wet weight at 30 days).

In conclusion, a method was developed to analyse quantitatively amino acids attached to tRNA in mycelia of *C. geophilum* by HPLC. Total cellular amino acyl-tRNA was extracted by a modified phenol extraction under conditions which were designed to prevent deacylation of the attached amino acids. After deacylation and separation from tRNA, fourteen amino acids were determined.

The determination of the state of charging of tRNA in vivo would allow the study of the possible participation of tRNA in the control of protein synthesis and breakdown in ectomycorrhizal fungi and ectomycorrhizae.

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